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Review

Cytochrome *bd* oxidase and nitric oxide: From reaction mechanisms to bacterial physiology

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Dedicated to the memory of Professor António V. Xavier

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ABSTRACT

Experimental evidence suggests that the prokaryotic respiratory cytochrome *bd* quinol oxidase is responsible for both bioenergetic functions and bacterial adaptation to different stress conditions. The enzyme, phylogenetically unrelated to the extensively studied heme–copper terminal oxidases, is found in many commensal and pathogenic bacteria. Here, we review current knowledge on the catalytic intermediates of cytochrome *bd* and their reactivity towards nitric oxide (NO). Available information is discussed in the light of the hypothesis that, owing to its high NO dissociation rate, cytochrome *bd* confers resistance to NO-stress, thereby providing a strategy for bacterial pathogens to evade the NO-mediated host immune attack.

1. Introduction

Cytochrome *bd* [1–3] is a respiratory terminal oxidase widely distributed among prokaryotes (Eubacteria and Archaea), not yet found in eukaryotic organisms. Identified more than 80 years ago [4], its impact on cell physiology is still not completely understood, which makes this respiratory enzyme an attractive system to be investigated. Beyond its role in energy metabolism, a large body of evidence suggests that the enzyme plays alternative functions relevant to physiology and, more specifically, to bacterial adaptation to a wide variety of stress conditions [3,5]. This respiratory oxidase has been identified in a number of pathogens [6–15] and, in some cases, its expression level was intriguingly found to positively correlate with virulence. Evidence suggests that cytochrome *bd* is implicated in adaptation to the hostile conditions created by host immunity during the infection process, though the molecular mechanism(s) through which the enzyme operates to enhance bacterial resistance have been only partly clarified.

Cytochrome *bd* shows no sequence homology with the extensively studied heme–copper oxidases (HCOs) [16–19]. Compared to these latter oxidases, it exhibits interesting similarities at the level of the catalytic mechanism (see below), but also some characteristic peculiarities. While HCOs invariantly comprise a high spin heme iron (heme a_3, o_3 or b_3) and a copper metal (Cu_B) in the active site, cytochrome *bd* does not have a copper cofactor, but only three hemes. Both HCOs and cytochrome *bd* catalyze the complete, four-electron reduction of O_2 to H_2O; cytochrome *bd*, however, can only use quinols as natural reducing substrates [2], whereas the large superfamily of HCOs comprises both cytochrome *c*- and quinol-oxidases [17]. HCOs and cytochromes *bd* are characterized by distinct energetic efficiencies. The overall reaction catalyzed by most cytochromes *bd* is indeed electrogenic [20], leading to proton motive force generation, but contrary to HCOs, is not associated to proton pumping [21], thereby resulting in a lower efficiency.

Abbreviations: NO, nitric oxide; HCO, heme–copper oxidase; CcO, mitochondrial cytochrome *c* oxidase; CO, carbon monoxide; DTT, dithiothreitol; Q_1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone

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Depending on the intracellular concentration, nitrogen monoxide (nitric oxide, NO) plays a key role in a wide range of physiological and pathological processes, such as vasodilation, platelet aggregation, neuromodulation, cell death and host immunity. Over and above these functions, NO is also a potent inhibitor of HCOs (reviewed in [22–25]). Extensive studies on mitochondrial cytochrome *c* oxidase (CcO) proved that the enzyme is promptly inhibited by relatively low NO concentrations, even in the presence of an excess of O_2. Inhibition is reversible and, accordingly, the O_2-reductase activity is completely restored if NO in solution vanishes by reacting with O_2 or scavenging systems (like oxygenated myoglobin/hemoglobin). Interestingly, the transient NO-inhibition of mitochondrial respiration at CcO has been suggested to control several important physiological processes (reviewed in [24–27]), including ATP synthesis, extra-mitochondrial O_2-dependent enzymatic reactions, tissue oxygenation, reactive oxygen species mitochondrial production and cell signalling.

CcO is inhibited by NO according to two different pathways [22–25]. NO can either bind to ferrous heme a_3 in the active site, yielding a stable nitrosyl adduct, or it can generate a nitrite-bound ferric heme a_3 adduct, by reacting with enzyme species containing fully oxidized heme a_3/Cu_B or ferryl heme a_3. In the latter case (nitrite pathway), NO was proposed to react with oxidized Cu_B leading to the transient formation of nitrosonium ion (NO^+), subsequently converted into nitrous acid/nitrite by hydroxylation [28–31]. The O_2-competitive nitrosyl pathway prevails at higher electron flux and lower O_2 concentration, whereas the nitrite pathway at lower electron flux and higher O_2 concentration [32]. In the former case, inhibition is persistent and, in the absence of free NO in solution, reverts at the low rate of NO dissociation form ferrous heme a_3 ($k = 0.0035\ s^{-1}$ at 20 °C, [32], see Table 1). Conversely, in the latter case, if NO in solution is exhausted, activity is promptly recovered following the fast nitrite ejection from heme a_3 upon reduction of this site [33].

In their natural habitat prokaryotes often encounter the NO produced from abiotic or biotic sources. Particularly, host infecting bacteria are challenged by the NO and related reactive species (collectively termed 'reactive nitrogen species') that are produced as part of the immune response to control microbial proliferation. Consistently, during evolution infectious microorganisms have developed systems to detoxify NO and survive nitrosative stress. NO detoxification in microbes is mainly accomplished by enzymes endowed with NO-reductase or NO-dioxygenase (O_2-denitrosylase) activity, such as the NO-reductase flavorubredoxin [34,35] and the flavohemoglobin [36,37] in *Escherichia coli*. NO-reductases anaerobically reduce NO to dinitrogen oxide (N_2O), while NO-dioxygenases aerobically degrades NO to nitrate (NO_3^-). Under aerobic conditions, flavohemoglobin scavenges NO with high efficacy, but the catalytic activity of the enzyme is characterized by a relatively low apparent affinity for O_2 ($K_M \sim 20$–$100\ \mu M\ O_2$, [38,39]).

Given the wide distribution of cytochrome *bd* in prokaryotes and its occurrence in several bacterial pathogens, recent studies aimed at investigating the reactivity of NO with this respiratory oxidase in order to assess its relevance to microbial physiology. The results from these studies are reviewed here in the light of the proposal that physiologically cytochrome *bd* can enhance bacterial tolerance to nitrosative stress, thereby promoting pathogenicity.

2. Catalytic intermediates

The three-dimensional structure of cytochrome *bd* is yet unknown. The enzyme is a heterodimer of two integral membrane polypeptides, subunits I and II, carrying three heme cofactors: the low spin hexacoordinate heme b_{558} and the high spin pentacoordinate hemes b_{595} and *d* [40] (Fig. 1). Heme b_{558} in subunit I represents the site of quinol oxidation, whereas heme *d* is the site where O_2 chemistry takes place. Conversely, the function of heme b_{595} is not yet fully understood. This redox site has been proposed to form together with heme *d* a bimetallic active site [41–49], thus functionally mimicking Cu_B in HCO. Beta-lactamase gene fusion experiments showed that all three hemes are likely located near the periplasmic space [50]. Based on this topology, membrane potential generation by cytochrome *bd* is expected to primarily result from proton transfer from the cytoplasm to the enzyme active site on the opposite side of the membrane, rather than from inter-heme electron transfer.

Insights into the catalytic intermediates of cytochrome *bd* were obtained by flow-flash investigation of the reaction of the fully

Table 1
Rate constants for the reactions of reduced CcO or cytochrome *bd* with O_2, CO and NO.

	Beef heart cytochrome *c* oxidase			Cytochrome *bd* from *E. coli* or *A. vinelandii*[1]					
	O_2	CO	NO	O_2		CO		NO	
	R^4	R^4	R^4	R^1	R^3	R^1	R^3	R^1	R^3
k_{on} ($M^{-1}\ s^{-1}$)	1.3×10^{8}[a]	8.0×10^{4}[b]	1.0×10^{8}[c]		**0.3×10^{9}**[f] **2.0×10^{9}**[g] 2.0×10^{9}[h] 1.9×10^{9}[i]	**1.0×10^{8}**[l]	**1.5×10^{8}**[l] 0.8×10^{8}[h,m]		
k_{off} (s^{-1})		0.023[b]	0.0035[d]	78[e]		4.2[e]	6.0[e]	0.036[e]	0.133[e]
K_d (nM)		300[n]	0.2[n]	280[p]			80[q]	0.55[r]	

[1] The values relative to the *A. vinelandii* cytochrome *bd* are depicted in bold.
[a] [97].
[b] [83].
[c] [98].
[d] [32].
[e] [58].
[f] [99].
[g] [54].
[h] [51].
[i] [52].
[l] [59].
[m] [49].
[n] [76].
[p] [57].
[q] [63].
[r] [77].

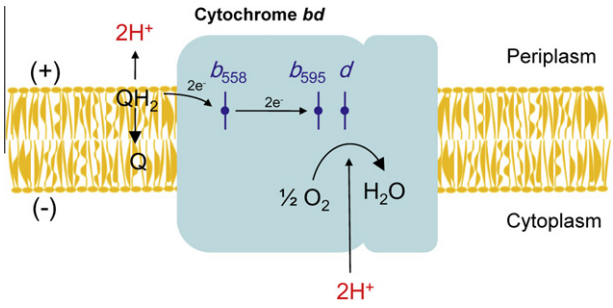

Fig. 1. Schematic representation of cytochrome *bd*. Electrons donated by quinols are transferred from heme b_{558} to heme *d* where the O_2 chemistry takes place. The function of heme b_{595} is still debated. The reaction is electrogenic, but not coupled to proton pumping.

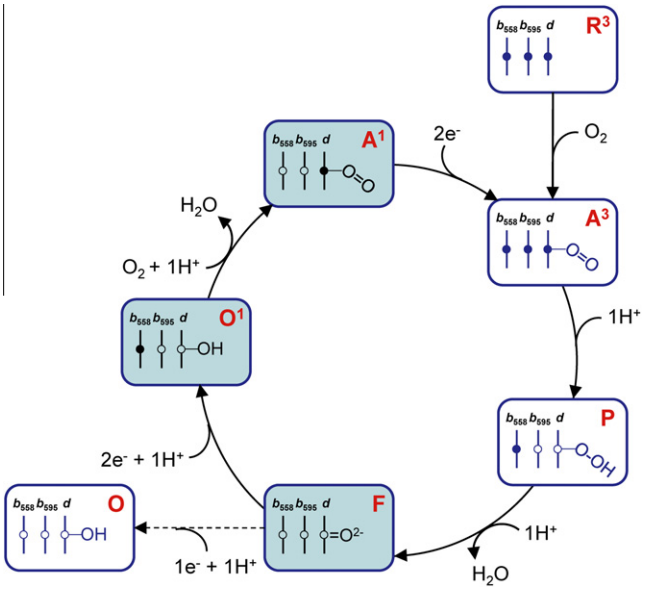

Fig. 2. Suggested catalytic cycle. The heme redox state in each intermediate is denoted by closed (Fe^{2+}) or open (Fe^{3+}) symbols. In the **F** intermediate, heme *d* is in the Fe^{4+} redox state. The intermediates detected at steady-state [60] are highlighted. The fully ferric (**O**) species was suggested not to be an intermediate of the catalytic cycle [61]. Contrary to the four-electron reduced CcO, in the absence of exogenous reducing equivalents, the three-electron reduced cytochrome *bd* (**R**) by reacting with O_2 yields the **F** intermediate as the final species [20].

(three-electron) reduced enzyme ($\mathbf{R^3}$ [1]) with O_2. In these studies, the reaction was monitored by spectroscopic or electrometric techniques [20,41,51–53], following laser photolysis of the carbon monoxide (CO)-bound $\mathbf{R^3}$ enzyme in the presence of O_2. These measurements show that cytochrome *bd* and HCOs share similarities at the level of their catalytic intermediates (Fig. 2). Likewise CcO and other HCOs, the first intermediate detectable after CO-photolysis is the so-called compound **A** ($\mathbf{A^3}$ in Fig. 2), i.e., the $\mathbf{R^3}$ enzyme with O_2 bound to ferrous heme *d*. Formation of $\mathbf{A^3}$ was shown to occur at high rates ($k_{on} \sim 2 \times 10^9\ M^{-1}\ s^{-1}$ [51,52,54]) without generating electric potential [20,52]. Afterwards, a rapid ($\tau = 4.5$ μs), non-electrogenic oxidation of heme b_{595} was observed and the resulting species was suggested to be a peroxy intermediate (**P**) [52,53]. Notably, in the reaction of CcO with O_2 such an intermediate is not populated at detectable levels, as it rapidly converts to a ferryl species, that anyway for historical reasons was named '**P**' (as incorrectly assigned to a peroxo-species) (see [55] and references therein). In the absence of exogenous electron equivalents, as the final step in the reaction, heme b_{558} in cytochrome *bd* is oxidized and the ferryl (**F**) intermediate is populated with $\tau = 47$ μs. This last **P**→**F** transition is electrogenic [20,41,52,53]. In enzyme preparations with bound quinol, an additional electrogenic phase was observed with $\tau = 0.6$–1.1 ms [41,52], assigned to the conversion of **F** into the oxidized enzyme (**O**) or, more likely, into the single-electron reduced, oxygenated enzyme ($\mathbf{A^1}$), since quinol can act as a two-electron donor.

Owing to the high affinity of heme d^{2+} for O_2 [54,56,57], the $\mathbf{A^1}$ species of cytochrome *bd* with ferrous-oxy heme *d* is stable so that a considerable fraction of the enzyme is isolated in such a state [54,56,57]. The high stability of $\mathbf{A^1}$ is a peculiarity of cytochrome *bd*. This species was not observed in HCOs, to which O_2 can only bind at the heme–copper binuclear site in the two-electron reduced state. The rate of O_2 binding to $\mathbf{R^1}$ cytochrome *bd* ($b_{558}{}^{3+}b_{595}{}^{3+}\ d^{2+}$) was directly measured by flow-flash and found to hyperbolically depend on O_2 concentration [54]. In contrast the $\mathbf{R^3}$ enzyme binds O_2 with rates linearly dependent on O_2 concentration [54], as expected for a simple bimolecular process assayed under pseudo-first order conditions. The saturation kinetic behaviour observed with $\mathbf{R^1}$, but not with $\mathbf{R^3}$, was tentatively explained by assuming that in the $\mathbf{R^1}$ enzyme two conformations, one O_2-accessible ('open') and the other O_2-inaccessible ('closed'), co-exist in equilibrium [54]. Consistently, also in the case of CO,

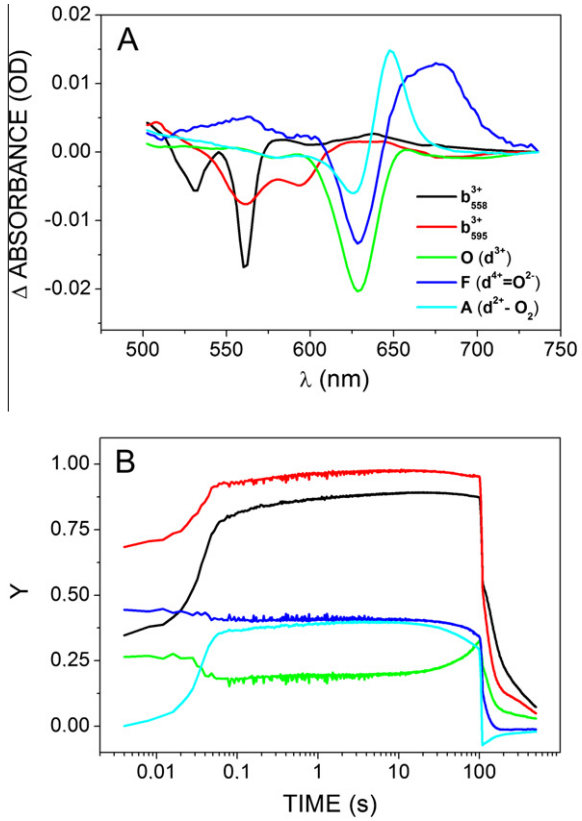

Fig. 3. Catalytic intermediates detected at steady-state. Panel A: Reference optical spectra (normalized to 1 μM enzyme): '$b_{558}{}^{3+}$' = [$Fe_{b558}{}^{3+}$] – [$Fe_{b558}{}^{2+}$]; '$b_{595}{}^{3+}$' = [$Fe_{b595}{}^{3+}$] – [$Fe_{b595}{}^{2+}$]; '**O**' = [$Fe_d{}^{3+}$] – [$Fe_d{}^{2+}$]; '**F**' = [$Fe_d{}^{4+}$ = O^{2-}] – [$Fe_d{}^{2+}$]; '**A**' = [$Fe_d{}^{2+}$–O_2] – [$Fe_d{}^{2+}$]. Panel B: Fractional occupancy of the optical species depicted in (A) as obtained from deconvolution analysis of the time-resolved spectra collected after stopped-flow mixing 20 μM enzyme (pre-reduced with 10 mM DTT and 600 μM Q_1) with O_2-equilibrated buffer (see [60] for details). Colour code as in (A). Please notice that part of the reaction of the reduced enzyme with O_2 is lost in the dead-time of the stopped-flow apparatus, followed by an oxidation phase (<100 ms), a steady-state phase (up to ~100 s) and after O_2 exhaustion a reduction phase eventually restoring the fully reduced enzyme.

[1] The superscript number denotes the total number of electrons in the enzyme species.

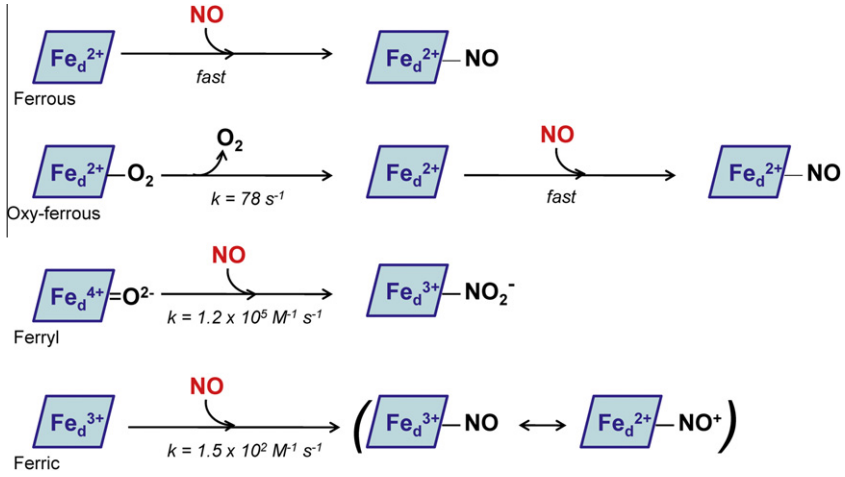

Fig. 4. Reactions of heme *d* with NO. According to [58,79], the reaction of NO with ferrous or ferryl heme *d* is fast and yields a nitrosyl ferrous (Fe^{2+}–NO) or a nitrite-ferric (Fe^{3+}–NO_2^-) derivative, respectively. A nitrosyl ferric (Fe^{3+}–NO↔Fe^{2+}–NO^+) adduct was instead suggested to form upon reaction of ferric heme *d* with NO [82].

higher on- and off-rates were measured for the **R**^{3} cytochrome *bd*, as compared to the **R**^{1} enzyme [49,51,58,59] (see Table 1). Ligand binding properties of heme *d* are therefore clearly influenced by the redox state of the heme(s) *b*, but the structural determinants of this redox control remain elusive.

Recently, the steady-state occupancy of the cytochrome *bd* catalytic O_2 intermediates during turnover was measured by stopped-flow multiwavelength absorption spectroscopy [60], taking advantage from the fact that the O_2-reactive heme *d* absorbs in the visible region at wavelengths (>600 nm), where the spectral contributions of the hemes b_{558} and b_{595} are minor (Fig. 3A). In that study, time-resolved absorption spectra were collected after rapidly mixing reduced cytochrome *bd* with O_2-equilibrated buffer in the presence of excess DTT (dithiothreitol) and Q_1 (2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone). Global analysis of the spectral data showed that the ferryl (**F**) and oxy-ferrous (**A**) species are the mostly populated catalytic intermediates at steady-state, with a residual minor fraction of the enzyme containing ferric (**O**) heme *d* and possibly one electron on heme b_{558} (Fig. 3B). This is in line with the proposal that the fully ferric species is not an intermediate of the catalytic cycle [61] (see Fig. 2). These results obtained with cytochrome *bd* differ from those obtained under similar conditions with CcO, using cytochrome *c* as the reducing substrate, where the steady-state occupancy of the oxygen catalytic intermediates was reportedly very low (<10%, [62]).

3. Reactions with nitric oxide

In the Cu-lacking *bd*-type oxidases, NO, as CO [63] and O_2 [54,57], binds to ferrous heme *d* with high affinity [64,65] (Fig. 4). Formation of the resulting nitrosyl adduct is accompanied by characteristic changes in the UV–visible absorption and electron paramagnetic resonance (EPR) spectra of the enzyme [64,65]. At low concentrations, NO almost exclusively binds to heme d^{2+}, whereas at high concentrations low-affinity NO binding to heme b_{595} [64,65] or heme b_{558} [46] was suggested too. The yet unknown rate of NO binding to the ferrous uncomplexed heme *d* is expected to be very high, and possibly comparable to that of O_2 ($k_{on} \sim 2 \times 10^9\ M^{-1}\ s^{-1}$ for **R**^{3} [51,52,54]). The reaction of NO with oxy-ferrous heme *d* (Fe^{2+}–O_2) in the **A**^{1} enzyme also results ultimately in formation of the nitrosyl heme *d* adduct after displacement of the O_2 bound, but in this case NO binding is limited by the off-rate of O_2 from the reduced heme ($k = 78\ s^{-1}$ at 20 °C, [58]) (Fig. 4).

Some bacterial HCOs [66–68], but not mitochondrial CcO [69], under anaerobic conditions exhibit a low NO-reductase activity, yielding N_2O as the final product. This result is perhaps not surprising, since HCOs are believed to share a common phylogenesis with heme b_3-containing bacterial NO-reductases [70,71]. Since the ability of some HCOs to function as NO-reductases was tentatively attributed to structural and/or functional peculiarities at the level of Cu_B [66], the lack of this metal in cytochrome *bd* raised the question as to whether the enzyme is capable of reductively metabolising NO to N_2O. Making use of a selective NO electrode, amperometric measurements carried out on cytochrome *bd* purified from *Escherichia coli*[2] or *Azotobacter vinelandii* clearly showed that neither of these two Cu-lacking oxidases is endowed with a measurable NO reductase activity [72]. Based on this finding, one may conclude that the presence of a non-heme metal in the active site (copper in HCO or iron in bacterial NO-reductase) is a prerequisite for reduction of NO; more recently, however, coordination of NO to Cu_B was proposed to be a dispensable step in the mechanism of NO reduction by HCOs [73].

Similarly to CcO, cytochrome *bd* is inhibited by NO. First evidence for this was provided by measuring the NO-sensitivity of cytochrome *bd*-mediated respiration by *E. coli* mutant cells lacking the alternative heme–copper cytochrome bo_3 quinol oxidase [74]. In agreement with these measurements, in the presence of DTT and Q_1, the O_2 consumption catalyzed by cytochrome *bd* isolated from *E. coli* or *A. vinelandii* is rapidly inhibited upon addition of relatively low amounts ($\leqslant 1\ \mu M$) of NO [72]. Possibly relevant to microbial physiology, the degree of inhibition depends on the O_2 concentration, the inhibition being more potent at lower [O_2]. The IC_{50} value for *E. coli* cytochrome *bd* inhibition by NO (100 ± 34 nM at 70 μM O_2 [72]) is not very different from that reported for CcO [75,76], and proved to be linearly dependent on [O_2] (at least up to ~150 μM O_2) in experiments carried out on cytochrome bo_3-lacking *E. coli* cells [77]. Interestingly, based on this information, inhibition of cytochrome *bd* is likely to occur in vivo during bacterial infection, when oxygen concentration is quite low and μM NO is produced by immunocompetent cells to counteract microbial proliferation.

All together, the studies mentioned above demonstrate that the presence of Cu_B is not essential for terminal oxidases inhibition by NO. The question arising is: what is the exact mechanism whereby

[2] In addition to the heme-copper cytochrome bo_3 quinol oxidase, *E. coli* possesses two *bd*-type oxidases, called *bd*-I and *bd*-II. Unless otherwise stated, we refer to cytochrome *bd*-I throughout the manuscript.

cytochrome *bd* in turnover with O_2 is inhibited by NO? One may speculate that inhibition proceeds through the reaction of NO with enzyme species containing ferrous uncomplexed heme *d*, owing to its high ligand binding affinity; heme d^{2+} can indeed be targeted by both O_2 and NO and this reasonably accounts for the observed O_2-competitive nature of NO-inhibition [72,77]. On the other hand, the fast inhibition of the enzyme observed at low NO concentrations ($\leqslant$1 μM) even in the presence of a large excess of O_2 (100–200 μM), commonly observed also with CcO, is somewhat puzzling. At this low NO/O_2 ratio, if NO was reacting solely with ferrous uncomplexed heme *d*, accumulation of the inhibited enzyme should be slower than observed; NO binding to heme d^{2+} would indeed be kinetically outcompeted by the O_2 present in large molar excess, since both ligands are expected to react with heme d^{2+} with similar second order rate constants. To account for this discrepancy, one may hypothesize that, similarly to CcO, the enzyme in turnover populates catalytic intermediates (without ferrous uncomplexed heme d^{2+}) that, in spite of their low or even absent reactivity towards O_2, are promptly targeted by NO leading to enzyme inhibition.

The hypothesis seems tenable, because we now know that the mostly populated catalytic intermediates at steady-state, the oxy-ferrous (**A**) and ferryl (**F**) intermediates [60], clearly O_2-unreactive, can both react with NO, though each one according to a different reaction pathway (Fig. 4). As mentioned above, reaction of $\mathbf{A^1}$ with NO ends-up with formation of the heme–NO adduct at a rate-limiting value of 78 s^{-1} assigned to the k_{off} of O_2 from the reduced heme *d* [58]. In contrast, working on the enzyme purified from *A. vinelandii*, the **F** intermediate[3] proved to react rapidly with NO, yielding the oxidized enzyme with nitrite bound at oxidized heme *d* [79]. The reaction occurs according to a 1:1 stoichiometry and proceeds at $k = 1.2 \pm 0.1 \times 10^5$ M^{-1} s^{-1} at 20 °C, i.e., it is even faster than the same reaction described for CcO ($k \sim 1$–2×10^4 M^{-1} s^{-1}), where Cu_B was suggested to be the primary site of the reaction [30,31]. In this regard, the results obtained on the Cu-lacking cytochrome *bd* suggest that Cu_B is not essential or possibly not even involved in the reaction. It remains to be established whether such a reaction (i) involves heme b_{595}, with this heme functionally mimicking Cu_B in heme–copper oxidases, or (ii) proceeds by direct reaction of NO with ferryl heme *d* iron, as documented with myoglobin and hemoglobin [80,81].

On the contrary, Cu_B is likely to facilitate the reaction of NO with ferric heme in the fully oxidized (**O**) active site of terminal oxidases. A remarkably different reactivity of the fully oxidized enzyme was observed in the Cu-lacking cytochrome *bd* [82], as compared to CcO [28,29]. In CcO, the reaction of the fully oxidized enzyme with NO is fast ($k = 2.2 \times 10^5$ M^{-1} s^{-1} at 20 °C, [29]) and was proposed to proceed through the following steps: (i) oxidation of NO to nitrosonium ion (NO^+) at Cu_B, (ii) hydroxylation of NO^+ to nitrous acid/nitrite and, eventually, (iii) nitrite binding to ferric heme a_3 [28]. The reaction is hindered by chloride bound at the oxidized heme a_3-Cu_B site [29]. In contrast, regardless of chloride being present, reaction of cytochrome *bd* in the **O** state with NO is much slower ($k = 1.5 \pm 0.2 \times 10^2$ M^{-1} s^{-1} at 20 °C) and does not yield nitrite-bound enzyme, but rather a nitrosyl adduct (heme d^{2+}-NO^+ or heme d^{3+}-NO, Fig. 4) [82].

It is interesting to notice that, similarly to CcO, cytochrome *bd* inhibition by NO is reversible and, accordingly, activity promptly recovers upon NO depletion [72]. Notably, when NO in solution is rapidly scavenged (as, for instance, by oxy-hemoglobin to nitrate), the reversal of inhibition is much faster in cytochrome *bd* than in mammalian CcO under similar experimental conditions [32]. Under high electron flux conditions, the activity recovery of CcO is indeed rate-limited by the slow dissociation of NO from ferrous heme a_3 ($k = 0.0035$ s^{-1} at 20 °C, [32]). Since a similar Fe^{2+}–NO adduct is likely to form in cytochrome *bd* upon NO-inhibition, one may anticipate that the faster activity recovery of the enzyme should correlate with a faster NO dissociation from ferrous heme *d*, compared to CcO. The rate of ligand dissociation from heme *d* was thus measured both for the fully reduced ($\mathbf{R}^3$-NO) and single-electron reduced enzyme ($\mathbf{R}^1$-NO) [58], and compared with available information on CcO (see Table 1). Measurements were performed by rapidly mixing in a stopped-flow apparatus NO-bound cytochrome *bd* with air-equilibrated buffer in the presence of an excess of oxy-myoglobin. Under these experimental conditions, the NO bound to heme d^{2+}, displaced by O_2, dissociates and rapidly ($k = 3$–4×10^7 M^{-1} s^{-1}) oxidizes oxy-myoglobin to met-myoglobin, which thus accumulates at the rate of the ligand dissociation, yielding characteristic absorption changes. At 20 °C, NO dissociates mono-exponentially from $\mathbf{R}^3$ and $\mathbf{R}^1$ cytochrome *bd* at $k = 0.133 \pm 0.005$ and 0.036 ± 0.003 s^{-1}, respectively [58] (Table 1). This result supports the view that the redox state of the *b*-type hemes, particularly heme b_{595}, controls the pathway and/or the kinetic barrier for ligand dissociation from the active site of cytochrome *bd*. Notably, the rate of NO dissociation from heme *d* in fully reduced cytochrome *bd* is remarkably higher (about 30 fold) than the off-rate of the ligand from ferrous heme a_3 of CcO under similar experimental conditions ($k = 0.133$ s^{-1} vs. $k = 0.0035$ s^{-1}, [32,58]). Consistently, CO dissociation from fully reduced cytochrome *bd* is also considerably faster than from the mitochondrial enzyme ($k = 6.0 \pm 0.2$ s^{-1} vs. $k = 0.023$ s^{-1} [58,83], see Table 1), in agreement with the hypothesis that in HCO Cu_B controls ligand escape from the nearby heme to the bulk phase [84].

The higher k_{off} value for NO measured for cytochrome *bd* is fully consistent with the observation that NO-inhibition reverts more rapidly in the case of cytochrome *bd* than in CcO [72], a result of physiological relevance (see below). More recently, the off-rate of NO from *E. coli* cytochrome *bd* was independently measured working on bacterial cell suspensions [77] and found comparable (0.163 s^{-1} at 35 °C) with that one (0.133 s^{-1} at 20 °C) previously measured with the isolated fully reduced enzyme [58]. Interestingly, the cytochrome *bd* NO off-rate value is higher than measured for most hemeproteins. It is comparable to the NO dissociation rate from soluble guanylate cyclase in the presence of GTP ($k = 0.18$ s^{-1}, [85]), but lower than the very fast NO off-rate more recently measured for cd_1-type nitrite reductases (up to 200 s^{-1}, [86]).

As discussed below, based on the high NO off-rate of *bd*-type oxidases, expression of these enzymes, instead of HCOs, was suggested to enhance bacterial tolerance to nitrosative stress, thereby possibly promoting bacterial pathogenicity [58,87]. The hypothesis was further supported also more recently [77].

4. Impact on microbial physiology

Cytochrome *bd* contributes to energy transduction and storage in the bacterial cell. Its electrogenic[4] quinol:O_2 oxidoreductase activity, though not coupled to proton pumping [21], is indeed linked to the generation of a proton motive force across the membrane that is used for ATP synthesis by the ATP synthase [20,21,41,52,53]. Apart from its role in cell bioenergetics, however, cytochrome *bd* accomplishes a number of additional functions of physiological relevance for the bacterial cell (Fig. 5). Acting as an O_2-scavenger, the enzyme protects from inactivation of O_2-labile enzymatic functions (such as nitrogenase in diazotrophic bacteria) (see Refs. [3,5] and references therein) and promotes the

[3] The metastable **F** species can be generated in vitro by adding excess H_2O_2 to the enzyme [44,78,79].

[4] A non-electrogenic cytochrome *bd* (called bd-II) was reported in *E. coli* [88].

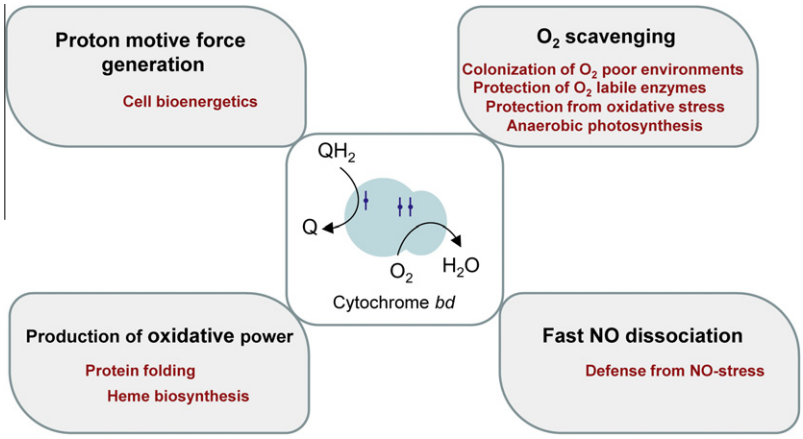

Fig. 5. Physiological functions proposed for cytochrome *bd*.

colonization of microaerobic environments inside the host by pathogenic and non-pathogenic bacteria [11,12,14,89]. Moreover, cytochrome *bd* was reported to expand the physiological range of environmental O_2 tensions at which anoxygenic bacterial phototrophs can grow [90]. The enzyme seems also to be implicated in the response of the bacterial cell to a wide range of stress conditions, that include low ambient O_2 tension, high temperature, high pH, membrane de-energization by uncouplers and oxidative stress (see Refs. [3,5] and references therein). Finally, *E. coli* cytochrome *bd*-I was proved to provide the oxidizing power required both by the DsbA–DsbB system to catalyze disulfide bond formation during protein folding [91] and by protoporphyrinogen IX oxidase (HemG), an enzyme involved in heme biosynthesis [92].

It is worth mentioning that the expression of cytochrome *bd* oxidases was documented in a number of bacterial pathogens, such as *Salmonella* [7,10], *Mycobacterium tuberculosis* [12], *Shigella flexneri* [8], *Streptococcus* [13], *Listeria monocytogenes* [15], *Brucella* [9,14], members of the strict anaerobe *Bacteroides* class [11], *Klebsiella pneumoniae* [6], and for some of these pathogens a correlation between enzyme levels and virulence was observed. Particularly interesting is the case of *Mycobacterium tuberculosis* in which a transient up-regulation of cytochrome *bd* was observed in vivo in the transition from acute to chronic infection of mouse lungs, along with a reduced virulence of a mutant strain defective in the cytochrome *bd*-associated transporter CydC [12]. In the same study, in vitro evidence was provided for stimulation of cytochrome *bd* expression in *M. tuberculosis* both under hypoxic conditions and, interestingly, in response to NO stress. All together, these data strongly suggest that the *bd*-type oxidase is required for *M. tuberculosis* adaptation to host immunity, playing a role in pathogenesis.

M. tuberculosis is not the only system in which stimulation of cytochrome *bd* expression by NO was documented. NO-induced expression of cytochrome *bd*-related genes was also reported for *Staphylococcus aureus* [93], *Bacillus subtilis* [94] and *Desulfovibrio gigas* [95]. Moreover, according to a transcriptomic analysis performed on chemostat-cultured *E. coli* [96], after exposure to NO, cytochrome *bd*-I genes proved to be preferentially induced, whereas unaltered expression of the alternative cytochrome bo_3 terminal oxidase was observed. This information nicely matches the finding that, in *E. coli*, compared to the heme–copper bo_3-type oxidase, cytochrome *bd* is less sensitive to NO-inhibition, i.e., it is characterized by higher IC_{50} values for NO, which in turn agrees with the observation that cytochrome *bd*-deficient *E. coli* cells exhibit a higher NO-induced growth inhibition, compared to cytochrome bo_3-deficient cells [77].

All this information suggests that cytochrome *bd* is implicated in the bacterial response to NO-stress. As originally proposed by our groups [58,87] and later substantiated [77], we think that the enzyme by virtue of its fast NO dissociation rate confers to bacterial cells a higher resistance to NO. One may indeed anticipate that the preferential expression of fast NO-dissociating cytochrome *bd* over HCOs under NO stress conditions could be beneficial for at least two reasons: it may (i) reduce the sensitivity of bacterial respiration to NO, if the *bd* enzyme is endowed with higher IC_{50} values for NO, and (ii) ensure a faster release of NO-inhibition, i.e., a faster recovery of respiration, in response to a decline in the NO flux. As pointed out by Mason et al. [77], the protective role of cytochrome *bd* from NO stress is expected to be particularly relevant physiologically under low O_2 tensions. Under these conditions, NO stability in solution is enhanced and the ability of the bacterial cell to detoxify NO is markedly reduced, due to the relatively low affinity of flavohemoglobin for O_2 [38], the main aerobic NO-scavenging enzymatic system in microorganisms. As the sites where bacterial infection takes place are typically O_2-poor, but enriched in the NO produced as part of the host immune response, a role for cytochrome *bd* in pathogenesis can be reasonably envisaged. Preferential expression of cytochrome *bd* may represent a strategy for bacterial pathogens to evade the host immune attack based on NO production.

5. Conclusions

Cytochrome *bd* oxidases are terminal oxidases confined to the prokaryotic world, phylogenetically unrelated to the better known HCOs. Extensive studies on the catalytic intermediates of cytochrome *bd* and their reactivity towards NO led to the discovery that the enzyme, though being promptly inhibited by this gaseous ligand at relatively low concentrations, quickly recovers activity upon NO removal from solution. The fast recovery has been attributed to a relatively fast NO dissociation rate from the active site of the enzyme, that is a peculiarity for a hemeprotein. The structural features accounting for this unusual behaviour are yet unknown, as the three-dimensional structure of cytochrome *bd* is currently not available. Experimental evidence, however, supports the hypothesis that the peculiar NO reactivity of cytochrome *bd* likely emerged during evolution as an adaptive strategy to enable bacterial survival under NO-stress conditions at low environmental O_2 tension. As conditions of this type are typically created by host immunity to counteract microbial infection, expression of cytochrome *bd* (instead of HCOs) in prokaryotic pathogens is expected to promote virulence. Consistently, correlations between cytochrome *bd* expression levels and bacterial virulence have been reported for specific pathogens. Studies on *bd*-type oxidases, however, need to be expanded to a wider range of bacteria to conclusively

demonstrate whether these respiratory terminal oxidases enhance pathogenicity by conferring a higher tolerance to nitrosative stress. These investigations may open new biomedical perspectives. If the role of cytochrome *bd* in bacterial pathogenicity is established, the identification of selective inhibitors could indeed pave the way to interesting pharmacological applications.

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